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Genital Papillomavirus in Greek Women With High-Grade Cervical Intraepithelial Neoplasia and Cervical Carcinoma

V. Labropoulou, E. Diakomanolis, S. Dailianas, K. Kalpaktsoglou, A. Rodolakis, S. Beaudenon, A. Kakkanas, and P. Mavromara

Molecular Virology Laboratory, Hellenic Pasteur Institute (V.L., A.K., P.M.) and 1st Department of Obstetrics and Gynecology, Colposcopy and Laser Surgery Unit, Athens University (E.D., S.D., K.K., A.R.), Athens, Greece; Unite des Papillomavirus, INSERM U190, Institut Pasteur, Paris, France (S.B.)

Fifty biopsies from high-grade squamous intraepithelial lesions (HG-SIL) and 14 cervical carcinoma biopsies from Greek women were screened for the presence of human papillomavirus (HPV) DNA sequences by Southern blot hybridization and by the polymerase chain reaction (PCR) for the presence of different HPV types. In high-grade SIL, HPV DNA sequences were detected in 44 of 50 biopsies with the following distribution: 36% HPV 16, 12% HPV 18, 6% HPV 31, 6% HPV 33, 4% HPV 51, and 24% unclassified HPV types. In cervical carcinoma biopsies, 13 of 14 specimens were positive for HPV DNA sequences. Six biopsies were positive for HPV 16, three were positive for HPV 18, and four contained unclassified HPV types. Overall, of the total 64 biopsies, 57 (89%) were positive for HPV DNA sequences. Of these, Southern blot hybridization alone detected HPV DNA sequences in 39 cases, whereas by PCR 18 additional specimens were found to be positive for HPV.

Among the HPV 16-positive biopsies, two samples produced a PstI banding pattern very similar but not identical to that of HPV 16 prototype and were referred to as HPV 16 isolates. One HPV 16 isolate appears to carry a mutation within the carboxy-terminal half of the L2 gene that results in the loss of a PstI site. The other HPV 16 isolate had a similar PstI banding pattern to that previously reported as HPV 16 "variant" found in Cape Town [Williamson et al., 1989, *Journal of Medical Virology* 28:146–149, 1994, *Journal of Medical Virology* 43:231–237.] and in Italy [Li Vigni et al., 1994, *2nd International Congress of Papillomavirus in Human Pathology* (Abstracts), p 100.].

KEY WORDS: human papillomavirus, Greek patients, cervical intraepithelial neoplasia, cervical cancer, HPV typing, Southern blot, PCR, RFLP analysis

INTRODUCTION

Human papillomaviruses (HPVs) are considered to be causally linked to the development of precancerous lesions of the cervix and cervical cancer [zur Hausen, 1989, 1994; Schiffman, 1992; Munoz et al., 1992]. More than 70 different HPV genotypes have not been described, and each is associated with a specific clinical entity [de Villiers, 1989]. Of these, ~30, including many that are still uncharacterized, are capable of infecting the genital tract; these HPVs have been further classified as either low-risk or high-risk types based on the preneoplastic nature of the clinical lesion with which they are associated. Low-risk HPVs, such as HPV 6 and 11, are most often associated with benign venereal warts and low-grade CIN lesions, which rarely progress into carcinoma. In contrast, high-risk HPVs, such as HPV 16 and 18, are found predominantly in high-grade CIN and cervical carcinoma [Bergeron et al., 1992; Lorincz et al., 1992; Lungu et al., 1992a; De Roda et al., 1994]. In general, HPV 16 represents the predominant type worldwide and together with HPV 18 have been detected in the 70% of human cervical cancers [zur Hausen, 1989]. Furthermore, HPV 18 is the predominant type in adenocarcinomas of the cervix and its presence is associated with rapid progression of the lesion [Smotkin et al., 1986, Kurman et al., 1988, Tase et al., 1988]. It should be noted that the epidemiological definition of low-risk and high-risk types correlates well with the transforming properties of the viral types as defined in vitro [von Knebel Doeberitz, 1992].

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Address reprint requests to P. Mavromara, Molecular Virology Laboratory, Hellenic Pasteur Institute, 127 Vas. Sofias Avenue, Athens Greece.

TABLE I. Occurrence of Different HPV Types in Greek Women With High-Grade SIL and Invasive Cancer*

HPV type	High-grade SIL (n = 50)	Invasive cancer (n = 14)	Total positivity (n = 64)
16	18 (36%)	6 (43%)	24 (37.5%)
18	6 (12%)	3 (21%)	9 (14%)
31	3 (6%)	—	3 (4.6%)
33	3 (6%)	—	3 (4.6%)
35	—	—	—
39	—	—	—
45	—	—	—
51	2 (4%)	—	2 (3.3%)
66	—	—	—
Unclassified	12 (24%)	4 (28%)	16 (25%)
Total	44 (88%)	13 (93%)	57 (89%)

*Results of the table are based on both Southern blot hybridization and PCR amplification. Two HPV 16, six HPV 18, one HPV 33, and 9 unclassified HPV types were found positive by PCR amplification only.

The incidence of HPV infection in various anogenital cancers is reported to vary in different geographical regions [Kjaer and Jensen, 1992]. Moreover, a significant variation has been described for the distribution patterns and frequency of certain HPV genotypes in different countries. For example, HPV 18 is usually the second most frequent type associated with cervical cancer, but it is of interest that there appears to be a significant geographical variation in its rate of distribution [Shah, 1992; De Roda et al., 1994]. HPV 18 was detected only in 4–5% of cervical cancer tissue samples from Colombia and Spain, China, India [Choo et al., 1987; Das et al., 1992; Munoz et al., 1992], but in 14–20% of tumor biopsies from Italy and the United States [Di Luca et al., 1987; Lorincz et al., 1992]. In contrast, HPV 18 does not always represent the second most prevalent type. For instance, in Belgium and Cape Town, the incidence of HPV 33 is much higher than that of HPV 18 [Williamson et al., 1989; Vandevelde et al., 1992], in Mali, HPV 45 has been reported as the second most prevalent type followed by HPV 18, and in Indonesia, HPV 18 represents the most common type followed by HPV 16 [Munoz, 1994]. Furthermore, in Japan, the incidence of both HPV 16 and HPV 18 is low [Yaegashi et al., 1990]. HPV 16 still represents the most common type but the incidence of other HPVs, such as 51 or 52, is much higher than that of HPV 18.

The aim of this study was to determine the incidence of HPV infection and the distribution of HPV types in biopsies from high-grade squamous intraepithelial lesions and cervical carcinoma among Greek women. For this purpose Southern blot hybridization and PCR amplification were applied using type-specific and general primers followed by RFLP analysis. This study represents the first report on the distribution of the high-risk genital HPV types in Greece.

MATERIALS AND METHODS

Study Population and Biopsy Specimens

The study consisted of 64 women whose ages ranged from 18 to 46 years. These women were referred to the Colposcopy and Laser Surgery Unit of Alexandra Hospital after an abnormal smear was found. The patients underwent a full colposcopic assessment using 5% acetic acid, and punch biopsies were taken from the most suspicious areas for histological diagnosis and HPV typing. All patients selected for this study presented high-grade squamous intraepithelial lesions (HG SIL) and were treated by laser CO_2 excisional therapy (laser cylindrical conization) [Diakomanolis et al., 1990].

Southern Blot Hybridization Analysis

Colposcopically derived biopsies were frozen immediately and total cellular DNA was extracted as described previously [Labropoulou et al., 1994]. The DNA samples (5–10 μg) were digested overnight with PstI restriction endonuclease (New England BioLabs), electrophoresed in duplicate 0.8% agarose gels, and transferred to BA-S85 (Schleicher & Schuell) nitrocellulose membrane according to Southern [1975]. Prehybridization was carried out overnight at 42°C in 5× SSPE, 1.5× Denhardt's solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA in the presence of 20% formamide (nonstringent conditions, Tm-40°C). Hybridization was carried out under the same conditions for ~48 hours using nick-translated ^{32}P-labelled vector-free probes (specific activity 1.2–2.5 × 10^8 cpm/μg) DNA. The probes were mixtures of the following HPV types: 6/11, 16/18/33, 31/35/39, and 45/51/66. After hybridization, the membranes were washed initially at room temperature with 2× SSC, 0.1% SDS and then at 48°C (low stringency conditions) and autoradiographed using Kodak X-Omat AR films for 1 day at −70°C. Subsequently, the filters were washed at 60°C in 1× SSC 0.1% SDS followed by a second wash in 0.1× SSC, 0.1% SDS (high stringency conditions) and exposed for 3–8 days. Each membrane was first hybridized using the 16/18/33 and 31/35/39 probe mixtures. The filters were then treated in 70% formamide at 65°C to remove the probes and each membrane was rehybridized under the same conditions using the 6/11 and 45/51/66 probe mixture, respectively.

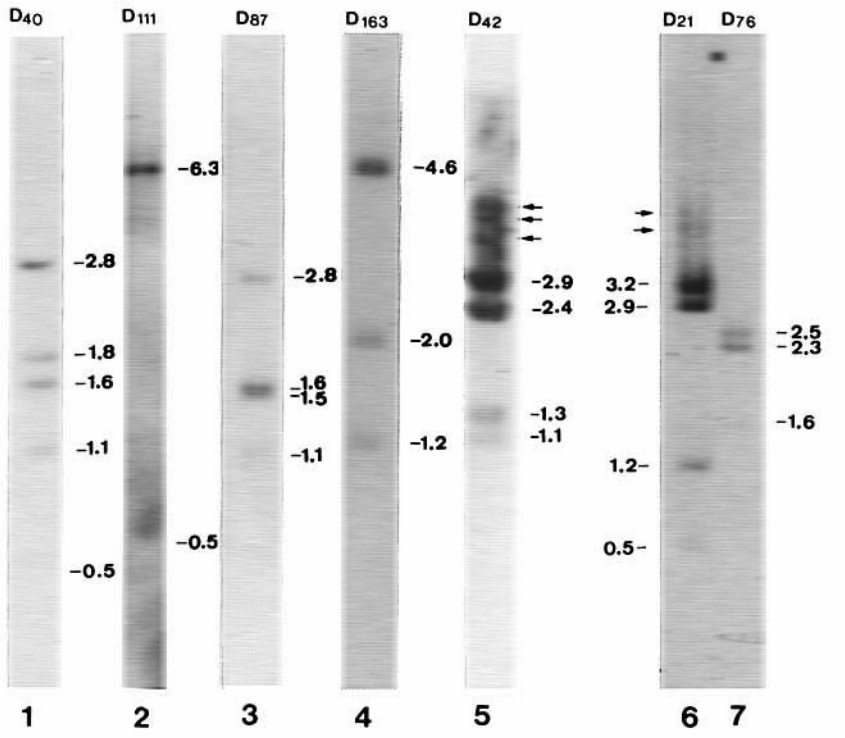

Fig. 1. Representative results of Southern blot analysis. Genomic DNA from biopsies of high-grade squamous intraepithelial lesions and cervical carcinoma were treated with PstI endonuclease, separated by electrophoresis in 0.8% agarose gel and transferred to nitrocellulose filter membranes. The membranes were hybridized under low stringency (Tm-40°C) conditions to ^{32}P-labelled mixed probes specific for HPV types 16/18/33, 31/35/39, and 45/51/66. Characteristic PstI patterns of HPV 16 **(lane 1)**, HPV 18 **(lane 2)**, HPV 31 **(lane 3)**, HPV 33 **(lane 4)**, and HPV 51 **(lane 5)**. Atypical PstI patterns of two uncharacterized HPV DNA samples from cervical carcinoma specimens detected by the HPV 45/51/66 probe mixture under low stringency conditions (**lanes 6** and **7**). The sizes of the PstI fragments for the specific HPV types are expressed in kilobases. Arrows indicate the extra PstI fragments present in the samples.

In a limited number of cases, confirmation of individual HPV types was done by hybridization under stringent conditions, i.e., 50% formamide, 5× SSPE, 1.5× Denhardt's solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA at 42°C using single probes.

Plasmid pHPI16-4 was constructed to carry the 1.9Kb BstNI-SphI (nt position 5528–7463) DNA fragment containing the L1 gene of HPV 16 cloned into the BamHI site of pUC19.

Polymerase Chain Reaction (PCR)

DNA preparations were analysed further by PCR using both type-specific and consensus (general) primer pairs. PCR amplification using type-specific primers was carried out with a mixture of primers designed to detect HPV types 6/11, 16, 18, and 33 as described previously [Riou et al., 1990]. The amplification products, a 306 bp fragment of the E4 region of HPV 6/11, a 185bp fragment of the E1 region of HPV 18, a 119bp fragment of the E6 region of HPV 33, and a 93 bp fragment of the E1 region of HPV 16 were analysed by electrophoresis in 3:1 NuSieve/agarose gels (FMC Corp., ME) followed by staining with ethidium bromide. Negative specimens were further analyzed by Southern blot hybridization using T4 kinase ^{32}P-labelled oligonucleotide probes. In cases where HPV typing was based on PCR results only, the specificity of the amplification products was tested by their cleavage pattern after HinfI restriction enzyme digestion.

In addition, PCR amplification using the consensus primers MY11 and MY09, designed to amplify a 450bp fragment of the L1 open reading frame from several HPV types, was used [Manos et al., 1989]. DNA was amplified as described previously [Ting and Manos, 1990] and the amplified products were analysed by 1.5% agarose gel electrophoresis and ethidium bromide staining. HPV genotyping of the consensus PCR amplification products was performed by digestion with a mixture of PstI, HaeIII, and RsaI (Biolabs) restriction enzymes as described previously [Ting and Manos, 1990; Lungu et al., 1992b]. PCR digested products were resolved by electrophoresis through a 4% MetaPhor agarose (FMC). Finally, a control set of primers (PC04, GH20) that amplifies a 268bp beta-globin gene fragment [Bauer et al., 1991] was included in the PCR analysis to assure the presence of an adequate amount of DNA target sequences. Oligonucleotides were synthesized by an Applied Biosystems DNA/RNA synthesizer Model 392.

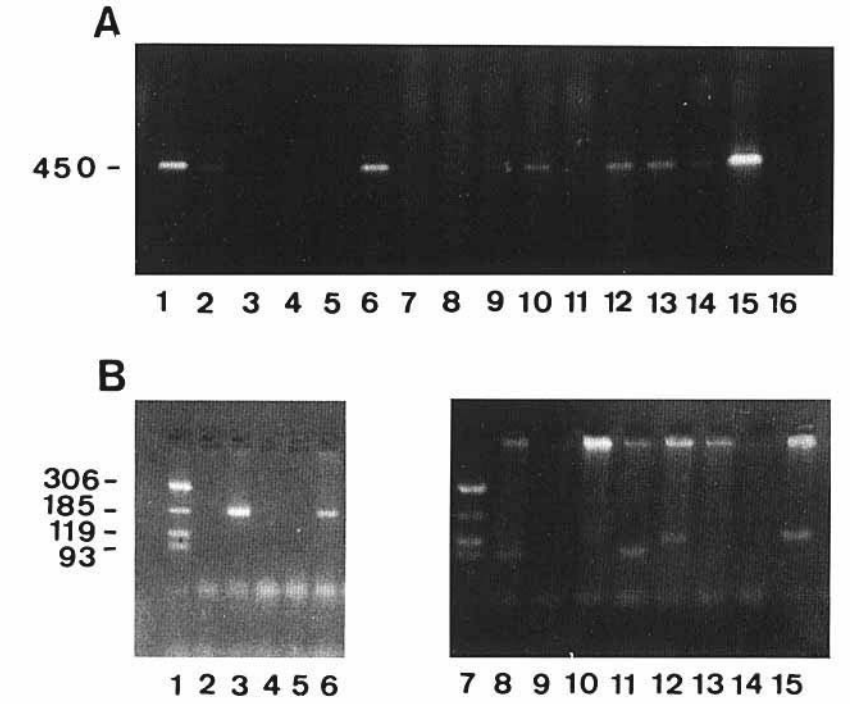

Fig. 2. PCR analysis. **A.** Ethidium bromide stained agarose gel (1.5%) showing the amplification product (450bp) of the general primer mediated PCR analysis using MY11 and MY09 consensus primers. **Lanes 1** through **14,** DNA preparations from biopsies. **Lanes 1, 2, 6, 10, 12, 13,** and **14** representative positive samples, **lane 15** positive control, and **lane 16** negative control. **B.** 3:1 NuSieve/agarose gel electrophoresis of the PCR amplification products using the type-specific primers. **Lanes 1** and **7** positive control consisting of a mixture of HPV 6/11, 16, 18, and 33 plasmid DNA producing amplification products of 306, 93, 185, and 119bp in size, respectively. DNA from biopsies positive for HPV 18 (**lanes 3** and **6**), HPV 16 (**lanes 8** and 11), and HPV 33 (**lanes 12** and **15**). The length of the amplified products are shown on the left.

RESULTS

In total, 64 colposcopically directed biopsies from Greek women were examined for the presence of HPV DNA sequences. Of these, 50 were diagnosed as high-grade squamous intraepithelial lesions (HG-SIL) and 14 as squamous cervical carcinoma. Initially, all biopsy DNAs were analysed by Southern blot hybridization using specific probes for 11 HPV genotypes (6, 11, 16, 18, 31, 33, 35, 39, 45, 51, and 66). Subsequently, PCR amplification was carried out for all specimens positive for beta-globin, using type-specific (6/11, 16, 18, 33) and consensus (MY09/MY11) primer pairs as described in Materials and Methods [Manos et al., 1989; Riou et al., 1990]. The results of the study are summarized in Table I. Specifically, HPV 16 type was detected in 18 out of 50 biopsies of high-grade lesions and in 6 out of 14 cervical carcinoma cases, whereas HPV 18 type was detected in six specimens of high-grade lesions and in three biopsies from cervical carcinomas. In high-grade squamous intraepithelial lesions, three biopsies contained HPV 31 type, three contained HPV 33 type, and two contained HPV 51 type. A total of 16 biopsies were found to contain unclassified HPV types, i.e., 12 specimens from high-grade lesions and four specimens from cervical carcinomas. Unclassified HPV types include specimens positive by Southern blot hybridization under low stringency conditions and/or positive samples by PCR amplification using the consensus primers that produced either low-yield PCR products or undetermined pattern in RFLP analysis. Of the 64 totally examined biopsies, 39 were positive both by Southern blot hybridization and PCR amplification, whereas 18 cases of the HPV-negative specimens by Southern blot analysis were found positive by PCR amplification only. These include two of the 24 biopsies positive for HPV 16, six of the nine biopsies positive for HPV 18, one of the three biopsies positive for HPV 33, and nine of the 16 biopsies that contained unclassified HPV types.

Figure 1 shows representative results from Southern blot analysis of the HPV types identified in this study, i.e., HPV 16 (D40), HPV 18 (D111), HPV 31 (D87), HPV 33 (D163), and HPV 51 (D42). The HPV genotypes were classified by analysis of the PstI banding patterns. In addition, the PstI pattern from the two "unclassified" HPV types found in the cervical carcinoma specimens by Southern blot analysis is also presented (Fig. 1, lanes 6 and 7). Hybridization in the latter case was carried out using the HPV 45/51/66 probe mixture, and the hybridization signals were detected only under low

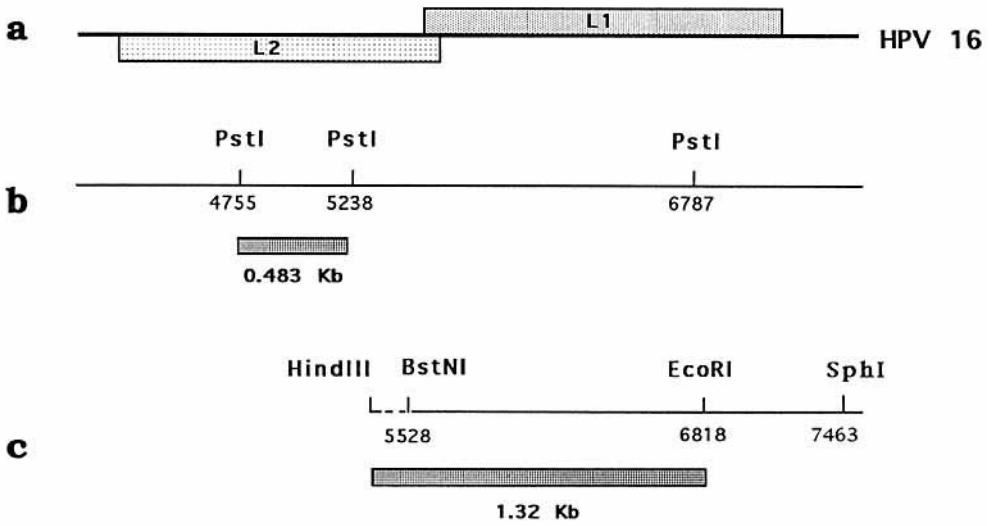

Fig. 3. Schematic representation showing the location of HPV 16 subgenomic probes used for the hybridization experiment presented in Figure 4. **a:** The region of HPV 16 genome that corresponds to L1 and L2 ORFs is represented by boxes. **b:** The position of PstI sites within the L1 and L2 genes of HPV 16. The filled box underneath indicates the location of the 0.5 Kb PstI-PstI fragment used as subgenomic probe. **c:** The position of the BstNI-SphI fragment from HPV 16, which has been cloned into pUC19 to yield pHPI16-4 plasmid. Underneath the filled box represents the 1.32Kb HindIII-EcoRI fragment used as probe. The dotted line represents pUC19 polylinker region.

stringency hybridization conditions and long exposure of the film. Both samples were positive by PCR analysis using the consensus (MY09/MY11) primers, whereas RFLP analysis of the amplification products produced uncharacterized patterns. It is of interest that in the case of the D21 specimen, the sum of the atypical PstI bands is ~ 8Kb, corresponding to the size of an intact HPV genome. Furthermore, digestion of the biopsy DNA with EcoRI restriction enzyme generated a single band of 8Kb for both specimens (data not shown). Further studies are in progress to determine whether these specimens contain "novel" HPV DNA sequences.

Figure 2 shows representative results of PCR amplification using the general primers (panel A) and the type-specific primers (panel B). We observed a complete agreement between HPV typing by Southern blot hybridization and PCR analysis using either type-specific primers or consensus primers followed by RFLP analysis.

Two cervical carcinoma biopsies showed a PstI banding pattern similar but not identical to HPV 16 prototype. As shown in Figure 4 (lane 2), the PstI cleavage of DNA preparation from specimen D54 gave two atypical PstI bands in addition to the expected 1.8Kb and 1.6Kb fragments; one of 2.7Kb, which is slightly smaller than the expected 2.8Kb band, and one of 1.2Kb, which is slightly larger of the expected 1.1Kb band. Further characterization of this HPV 16 isolate was not possible due to the limited amount of the specimen. It is of interest that this HPV 16 isolate has also been described by other investigators in other countries [Williamson et al., 1989, 1994; Li Vigni et al., 1994; Beaudenon, pers. comm.].

Treatment of the biopsy D162 DNA with PstI yielded in addition to 2.8Kb, and 1.1Kb fragments, two extra bands of 3.5Kb and 2.0Kb in size, whereas the expected 1.6Kb and possibly the 0.5Kb PstI fragments were missing (see Fig. 4, lane 4). Southern blot analysis of DNA preparation from a scrape specimen taken from the same woman gave exactly the same results. The 3.5Kb fragment was of low intensity and failed to hybridize when the membrane was rehybridized under high stringency conditions with HPV 16 alone (data not shown) suggesting the presence of different HPV sequences. In regard to the 2.0Kb fragment, we noticed that the size of this fragment corresponds approximately to the sum of the 1.6 and 0.5Kb PstI missing fragments and is most likely due to the loss of the PstI site.

To analyse this hypothesis further, the membrane was successively dehybridized and rehybridized using two different HPV 16 subgenomic probes. In the first series of hybridization experiments, the 1.32Kb HindIII/EcoRI fragment from plasmid pHPI16-4 was used as probe (Fig. 3). This fragment lies within the 1.6Kb PstI fragment that is absent in biopsy D162. As shown in Figure 4, the probe hybridized to the 1.6Kb PstI fragment of the HPV 16 prototype digests (lane 5), whereas in the case of the HPV 16 isolate, the probe hybridized to the 2.0Kb novel fragment (lane 5). In the second series of hybridization experiments, the 0.5Kb PstI fragment (nucleotide positions 4755–5238) derived from the cloned HPV 16 DNA was used as probe. This PstI fragment is adjacent to the 1.6Kb PstI fragment in the prototype HPV 16 DNA and lies within the L2 gene (Fig. 3). Hybridization results showed that the 2.0Kb fragment of the HPV 16 isolate also hybridized with this probe (Fig. 4, lane 8). These results indicate that the novel 2Kb PstI fragment from the biopsy D162 contains DNA sequences homologous to both the 1.6Kb

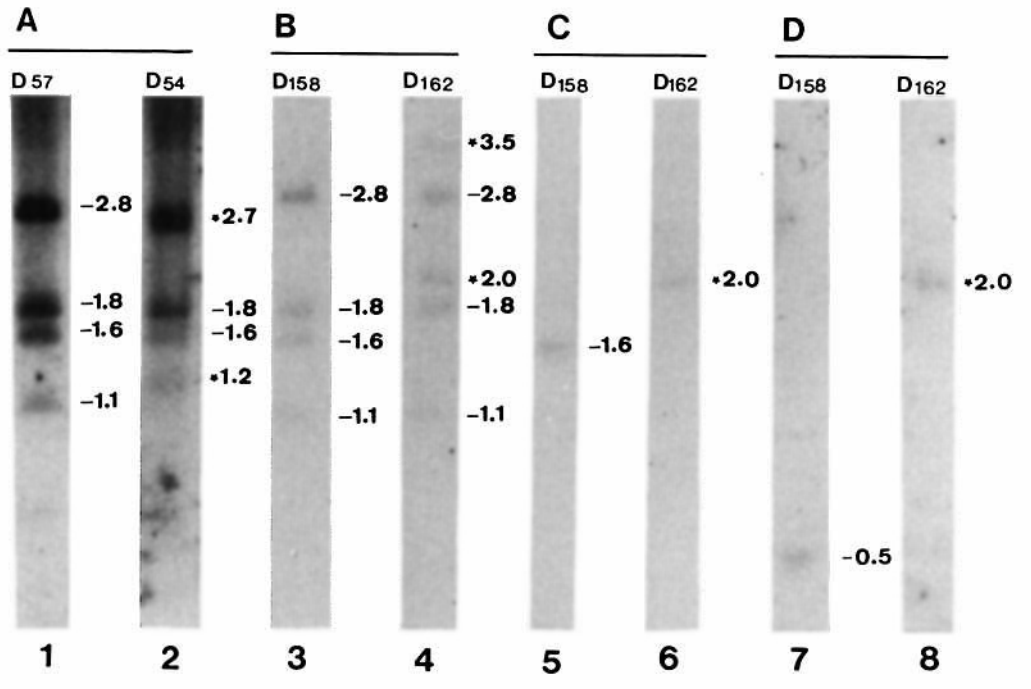

Fig. 4. Southern blot hybridization analysis of DNA preparations from cervical carcinoma biopsies containing two HPV 16 isolates. PstI digested DNA was electrophoresed in 0.8% agarose, transferred to nitrocellulose membrane, and hybridized to 16/18/33 probe mixture (**A** and **B**). Biopsies D57 (**lane 1**) and D158 (**lanes 3, 5,** and **7**) contain prototype HPV 16 DNA and serve as controls. Biopsies D54 (**lane 2**) and D162 (**lanes 4, 6,** and **8**) represent the HPV 16 isolates found in this study. The blots shown in B were sequentially hybridized with the 1.32Kb HindIII/EcoRI fragment from pHPl16-4 (**C, lanes 5** and **6**) and the 0.5Kb PstI fragment of HPV 16 (**D, lanes 7** and **8**). The size of the PstI fragments expressed in kilobases are shown on the right. Asterisks represent PstI fragments with abnormal mobilities.

and the 0.5Kb PstI bands of the prototype HPV 16 DNA, and its size corresponds to the sum of these PstI fragments. Taken together, these results suggest that the HPV 16 isolate from biopsy D162 has most likely lost the PstI site at position 5238 within the carboxy-terminal half of the L2 open reading frame. This would result in the loss of the individual 1.6Kb and 0.5Kb PstI fragments and the generation of a novel 2Kb PstI band homologous to both the 1.6Kb and the 0.5Kb DNA fragments. The PCR amplification using type-specific primers for HPV 16 gave positive results for both D54 and D162 specimens.

DISCUSSION

The rate of HPV detection and the distribution of HPV types in biopsies from high-grade squamous intraepithelial lesions and squamous cervical carcinoma of Greek women were analysed in this study. HPV-related DNA sequences were detected in 88% of high grade lesions and in 93% of cervical carcinoma biopsies. In agreement with the majority of the studies reported from other countries, HPV 16 was found to be the dominant type and the second most frequent type was HPV 18 [Williamson et al., 1989; Yaegashi et al., 1990; Bergeron et al., 1992; Das et al., 1992; Lorincz et al., 1992]. Interestingly, the prevalence rate of HPV 18 was found to be high both in high-grade and squamous cervical carcinoma cases. Other HPV types such as HPV 31, 33 and HPV 51 were less frequently found, whereas HPV types 35, 39, 45, and 66 were not detected in the material of this study. This differs from other studies where HPV 35, 39, and 45 are usually present at low frequency [Bergeron et al., 1992; Lorincz et al., 1992]. Unclassified HPV DNA sequences as determined by the PstI patterns in Southern blot and/or uncharacterized patterns after RFLP analysis of the PCR amplification product using the general primers were detected in high rate [Beaudenon et al., 1987; Tawheed et al., 1991; van den Brule et al., 1992]. Finally, seven cases had no HPV DNA sequences detectable by Southern blotting and PCR amplification. HPV-negative high-grade intraepithelial lesions and invasive cervical neoplasms have been reported in many studies worldwide. It has been suggested that they may portray a biologically distinct entity [Riou et al., 1990] or alternatively that these samples may contain low copy viral DNA or as yet unclassified HPVs [zur Hausen, 1989]. It is of interest, however, that a recent epidemiological study from The Netherlands, which is based on PCR analysis that allows the detection of a broad spectrum of HPV genotypes, reports a reduction in the percentage of HPV negatives in high-grade lesions and finds a low percentage of unidentified HPV types [De Roda et al., 1994].

In our study multiple infections with the common HPV types were not apparent even under low stringency hybridization conditions. However, in a limited number of cases, extra bands were observed in Southern blots that could represent infection with more than

one HPV type or alternatively integration of viral DNA into the host genome. The limited amount of DNA preparations and/or the low viral copy number did not allow further investigation. It has been suggested that viral DNA integration may represent a mechanism responsible for the progression of cervical cancer. However, recent studies failed to detect evidence of HPV integration in at least 30% of the analysed HPV-positive cancers, suggesting that integration may not be the unique prerequisite for malignant transformation [Choo et al., 1987; Cullen et al., 1991]. Nevertheless, HPV 18 DNA integration appears to occur in a much higher frequency [Cullen et al., 1991; Berumen et al., 1994].

The detection of two HPV 16 isolates both found in biopsies from patients with cervical carcinoma is also reported. Analysis of the PstI digestion pattern of biopsy D54 DNA gave a pattern similar to the HPV 16 "variant" found in several biopsies from Cape Town [Williamson et al., 1989, 1994]. The detection of a similar HPV 16 isolate was reported in four Italian patients [Li Vigni et al., 1994] and in France (Beaudenon, unpub. results). These findings indicate that this HPV 16 isolate is present in two geographically extreme locations, Africa and Europe. The second HPV 16 isolate designated D162 appears to carry a mutation that results in the loss of the PstI site at position 5238 within the carboxy-terminal half of the L2 gene. It should be noted that a considerable number of HPV 16 "variants" have recently been described. A remarkable genetic diversity characterized by genome rearrangements or point mutations has been reported for the noncoding region (NCR) [Kennedy et al., 1987; Tidy et al., 1989; Fang et al., 1993]. Moreover, single nucleotide changes have been observed within the L1 and L2 structural genes and in particular in the carboxyl-terminal half of the L2 gene [Icenogle et al., 1992; Pushko et al., 1994]. The biological significance of the HPV heterogeneity is not yet known. It has been hypothesized that certain HPV variants may be more aggressive and thus associated with rapid progression of the disease [Xi et al., 1993]. More studies are required to evaluate the importance of the great diversity of the genital HPVs in cervical pathology.

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